EFFICIENT SEEDING OF COLON CANCER CELLS TO DEVELOP AN *IN VITRO* THREE-DIMENSIONAL MODEL FOR DRUG EFFICACY STUDIES

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ABSTRACT

The project goal was to study different seeding techniques, static and dynamic seeding, to efficiently seed colon cancer cells onto a 3D matrix so that there was a uniform distribution of cells throughout the matrix with high cell density. Seeding the cells onto the matrix was done to develop a 3D *in vitro* model of colon cancer. Within the two methods, different variables were tested. The variables either pertained to the treatment of the matrix itself, the concentration of cells seeded to the matrix, or the hydrodynamics of the environment in which the cells and matrix are in.

For static seeding, it was observed that as time elapsed from the point of cellular inoculation, the percentage of cellular attachment to the scaffold initially showed an exponential phase, but after six hours the attachment of cells flat-lined. The highest percent attachment observed was 61%. Utilizing first-order kinetics, it was determined that the rate of attachment for the concentration range of 0.5 to 7 million cells was consistently about 0.15 hr^{-1}, indicating that within this range the rate was independent of the seeding concentration. Above this range the rates decreased by 33% or more.

For dynamic seeding, it was observed that the effects of time on cellular attachment was qualitatively similar to that of static seeding, but quantitatively different. At any given time, the percentage of cells attached would be higher in dynamic seeding than in static seeding. Unlike static seeding, 100% of the cells introduced to the system attached to the scaffold, and dynamic seeding showed an increase in the attachment rate when the initial seeding concentration was increased. Although it was observed that there was an increase in the rate of attachment as the initial seeding concentration increased, extrapolating from the data indicated that the rate of attachment asymptotically approached 0.285hr^{-1}. Therefore, the rate of attachment becomes independent of seeding concentrations at highly concentrated seeding levels. It was also observed that when the RPM of the system was increased, cellular attachment was reduced due to the increased shear stress supplied by the increase in the RPM.

Although the main focus was on the different seeding methods, scaffold properties were also investigated. It was observed that a high porosity and hydrophilic scaffold improved both the quantity of cells that attached and the rate of attachment of cells by 140%.

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INTRODUCTION

On average, it takes ten to twelve years and $900 million dollars for a pharmaceutical company to fully develop a drug that can be marketed. Typically, a pharmaceutical company begins with about 5,000 different compounds that are identified as potentially effective drugs but usually only one of these drugs will ever make it to the market. In order to increase productivity and reduce some of the costs incurred by a pharmaceutical company during drug development, it is critical to have an accurate model for drug testing during the preclinical trials. For instance, the use of a three-dimensional *in vitro* model instead of a simple two-dimensional model, will increase the accuracy and productivity of preclinical trials. Three-dimensional models are currently being used in tissue engineering to better represent *in vivo* tissue characteristics.

To produce a credible *in vitro* model of any biological tissue, the model should maintain the same structure, mechanics, and functions as its *in vivo* counterpart. In a biological system, an extracellular matrix (ECM) provides tissues with a characteristic structure, mechanics and some of its functions. An ECM has two main purposes. First, an ECM provides binding sites and a support structure for cells so that a specific shape can be obtained and maintained. Second, an ECM provides an environment for cells to transmit signals to regulate the activities of the tissue.

A simple two-dimensional model cannot duplicate any of the desired characteristics that an ECM provides. Thus, a three-dimensional scaffold is needed to provide an environment in which cells can grow and develop in the same manner in which it would in a natural environment. A three-dimensional scaffold would allow cells to develop into a tissue that has a length, width and depth.

In order to create a valid *in vitro* model of colon cancer for drug efficacy testing, colon cancer cells should be seeded in a three-dimensional environment. Consequently, the goal of this project was to study different seeding techniques in order to efficiently seed colon cancer cells onto a three-dimensional matrix in a timely manner such that there exists a uniform distribution of cells throughout the matrix at a high cell density. The purpose of seeding the cells onto a matrix was to develop a credible three-dimensional *in vitro* model of colon cancer.

Two different seeding methods were investigated: static and dynamic seeding. Within the two methods, different variables were tested. These variables either pertained to the treatment of the matrix itself, the concentration of cells seeded to the matrix, or to the hydrodynamics of the environment the cells and matrix were in.

Cells seeded to a three-dimensional matrix can be in one of three different situations: the seeded cells can become attached to the fibers of the matrix, the cells can be intercepted by the matrix, or the cells can be neither attached nor intercepted by the matrix. Attachment of the cells occurs when there is both an adequate surface area for contact and enough contact time between the cells and the fibers of the matrix. Attached cells are those cells that adhered to the fibers of the matrix. Adhered cells are unlikely to become separated from the matrix fibers by simple perturbations to the cells' immediate environment.

Unlike attached cells, intercepted cells do not adhere to matrix fibers. Intercepted cells occur when the cells are simply held in place due to the physical size of the pores on the matrix. These cells tend to clump together and when these clumps of cells become too big to pass between fibers they simply are intercepted and not attached to the

scaffold. Also, unlike attached cells, intercepted cells are highly susceptible to being easily displaced due to perturbations to the cells' immediate environment. Although intercepted cells do not attach readily, given enough time they could eventually attached.

The last situation that can occur to cells seeded to a matrix is that the cells can be neither attached nor intercepted by the matrix. These cells are no longer contained within the matrix because through certain driving forces, these cells have found their way out of the matrix. Consequently, these cells do not attachm unless they are reintroduced to the matrix.

LITERATURE REVIEW

Although research on the development of a three-dimensional *in vitro* model of cells may not be as extensive as that of other fields, several important studies have been done that make three-dimensional modeling a topic of great interest.

Alexis Carrel was the first scientist to introduce the use of three-dimensional *in vitro* cell modeling. Carrel used a silk veil as a scaffold for cells to attach to and develop in three-dimensions. The silk veil provided certain structural needs for the cells to develop into a three-dimensional tissue.[3]

Following Carrel's groundbreaking research in three-dimensional *in vitro* tissue modeling, the scaffold types were developed to provide for better cell attachment and growth and to more properly represent *in vivo* characteristics of tissues. Examples of scaffolds that have been tested are sponge matrices, collagen gels, filters, meshes, poly(lactic-co-glycolic acid) (PLGA), and poly(ethylene terephthalate) (PET).

Despite the diverse types of scaffolds used in developing *in vitro* three-dimensional models, scaffolds must possess basic properties that need to be optimized for each individual scaffold and cell combination. For example, studies have shown that the diameter of the fibers composing a scaffold needs to be larger than the diameter of the cells that are to be attached to the scaffold. This basic property must be satisfied because if the diameter of the fibers is too small, the curvature of the scaffold fiber surface area will not be adequate for the cells to properly adhere[12]. Since a scaffold physically supports the cells attached to it, the pore size and the alignment of fibers in the scaffold are additional parameters that affect cell attachment.[2]

A three-dimensional *in vitro* model differs significantly from a two-dimensional model. In three-dimensional models, cell-cell interactions, cell-ECM interactions and gene expression are all present. These attributes, which are very important to the characteristics of a tissue, are not entirely present in a two-dimensional model. The interactions in three-dimensional models become critical when drug tests are performed on the tissues. For instance, the presence of cell-cell interactions affects how resistant the cells are to the tested drug. The lack of this interaction would greatly underestimate the resilience of the cells to a certain drug concentration. Besides showing a more accurate drug resistance, three-dimensional *in vitro* models also show *in* vivo-like gene expression. It has been noted that some tumor antigens are only expressed in three-dimensional models and not in two-dimensional ones[7].

Figure 1 shows the apoptotic death mechanisms of cells in both a three-dimensional ECM environment and a non-ECM environment similar to that found in a two-dimensional culture. As seen in the top left-hand corner of Figure 1, the cells can either cluster together, unified with an ECM or remain secluded. The horizontal arrows indicate the path that is taken if the cells are to develop with an ECM, while the vertical arrow shows the path that is followed if cells are to remain independent. Depending on the natural or experimental conditions, the cells that are incorporated in an ECM are less susceptible to apoptosis, or programmed cell death, than cells outside of the three-dimensional ECM environment. It is also important to note that even though certain cells may be surrounded by cells that are part of the ECM, they are not directly linked to it and therefore are equally as susceptible to apoptosis as their isolated cell counterpart[10].

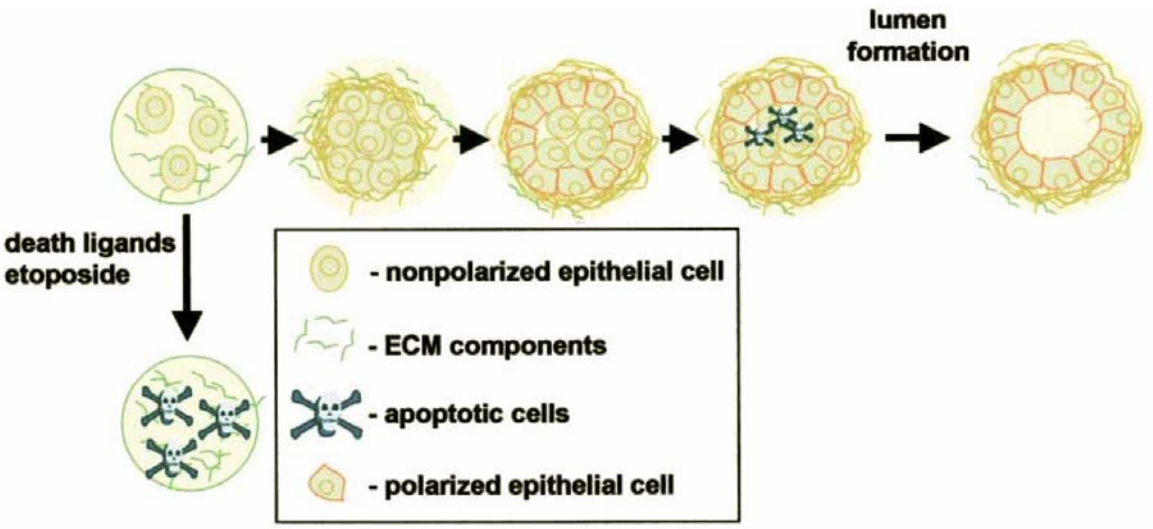

Figure 1: Apoptosis of Cells[10]

MATERIALS AND METHODS

Colon Cancer Cells:

HT-29, a human adenocarcinoma, grade I cancer cell type, was the cell line used for all of the experiments. This cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA). HT-29 has an epithelial morphology. HT-29 was chosen to be the particular cell line for these studies because it is widely used in many cancer studies.

Media:

To maintain the livelihood of the HT-29 cells, a specific type of media was used to supply nutrients for the cells to grow and divide. This media consisted of McCoy's 5A medium with L-glutamine and Dulbecco's Modified Eagle Medium (DMEM) with high glucose concentration, L-glutamine, and sodium pyruvate (GIBCO, Grand Island, NY). The DMEM was supplemented with 10% fetal bovine serum (GIBCO). The underlining difference between McCoy's medium and DMEM is the level of glucose concentrations. The levels of glucose concentrations for McCoy's medium and DMEM are 3.0 g/L and 4.5 g/L, respectively.

Matrix/Scaffold:

The material used as the scaffold in these experiments was poly(ethylene terephthalate) (PET), also known as Dacron.[4] The PET was non-woven and randomly arranged. PET was used because it creates an environment in which the cells can attach, grow in three dimensions, and form an extracellular matrix, much like it would *in vivo*.

The PET has a fiber diameter, density, and porosity of 20 μm, 1.35 g/mL, and 0.93, respectively.

To make the scaffold a more receptive environment for cells to attach, it was boiled in 1% NaOH for one hour. Boiling of the PET in 1% NaOH solution added additional functional groups to the scaffold making it more hydrophilic.[5]

Cell Counting:

A hemocytometer and a microscope were used to determine the number of cells in a particular sample. In most cases, the sample of interest was diluted by trypsan blue. This dilution was done for two reasons. The first reason for diluting the sample with trypsan blue was because the accuracy of the hemocytometer is bounded by an upper limit of the number of cells it can account for in a certain sample. If the sample being analyzed has a cell count higher than 300, then the hemocytometer cannot accurately determine the number of cells in that sample. The second reason for diluting the sample with trypsan blue was because it provides an indication of which cells are alive and which ones are dead. Since the dead cells stain blue, they can be observed and omitted in the cell count.

After the samples were properly diluted with trypsan blue, 10μl of that sample was injected into the opening of the hemocytometer and put under a microscope. While viewed under the microscope, the hemocytometer was divided into nine squares, three rows and three columns of squares. The corner squares were the only squares that counted. After all cells that resided in the four of the corner squares were counted and summed together (c), the number of cells in the sample (N) was determined by using **Equation 1.**

$$N = 2500*c*D*V_s \quad \textbf{Equation 1}$$

Where D is the dilution rate and V_s is the volume of the sample of interest.

Static Seeding

A variety of different variables were tested using the static seeding technique. The variables either pertained to the treatment of the scaffold itself or the concentration of cells initially seeded to the scaffold. Despite the many variables that were tested, the same basic procedure was followed to obtain results that could be compared.

Each experiment began by placing the scaffold in a six-well plate. The plate was filled with enough media to entirely cover the scaffold and incubated overnight at 37^{o}C. After the media was removed via pipette, the cells were then seeded on top of the scaffold by a new pipette. The seeding concentrations were varied depending upon which variable was being studied. After the cells were seeded onto the scaffolds, the six-well plates were placed into a 5% CO_2 incubator at 37^{o}C. For the following seven hours, the rate of cell attachment was monitored.

The cell attachment rate was monitored by removing the six-well plates from the incubator and gently rinsing two scaffolds every hour with phosphate buffered saline (PBS). The rinsed scaffolds were properly disposed of and the PBS was collected and put aside. Next, 3 mL of Accutase (Innovative Cell Technologies, San Diego, CA), used to detach cells from surfaces, was added to the wells that the two scaffolds recently resided in and then the six-well plates were placed back into the incubator. After 10 minutes, the 3 mL of Accutase were removed with a pipette and added to the PBS that was used for the scaffold washes. The cells were suspended in the solution of PBS and

Accutase by mixing and a sample of it is used to count the number of cells that at present in that sample. The hemocytometer was used to count the cells in the sample. The cells present in the PBS and Accutase solution are the cells that have not yet successfully been attached to the scaffold.

Dynamic Seeding:

Two variables were tested using the dynamic seeding technique. The variables tested either pertained to the concentration of cells initially seeded or to the environment that the dynamic seeding was taking place in. Despite the different variables that were tested, the same basic procedure was followed to obtain results that could be compared.

To begin the experiment set-up, the insides of the spinner flask were coated with Sigmacote (Sigma, St. Louis, MO), which is used to prevent cells from attaching to the walls of the spinner flask during the trials. The spinner flask is left over night so that the sigmacote can dry. While waiting for the Sigmacote to dry, the PET scaffold has to be sewed onto a mesh wiring so that the scaffold can be suspended inside the spinner flask while avoiding the spinner itself. After the Sigmacote had dried, the spinner flask was rinsed out and filled with 50 mL of de-ionized water. The PET scaffold that was sewn onto a mesh wiring was properly placed into the spinner flask. Once the autoclave was finished, the de-ionized water was poured out and 50 mL of media was added to the flasks. The spinner flask with the PET scaffold and the 50 mL of media was placed into the incubator for 24 hours.

The next day, the cells were seeded by a pipette into the 50 mL of media contained inside the spinner flask. A 0.5 mL sample of the media was taken so that an official initial seeding concentration could be measured. Then it was placed in the

incubator where a magnet rotated the spinner at a predetermined RPM. The concentration of the seeded cells varied depending upon which variable was being studied. For the next 10 hours, the rate of cell attachment was monitored.

Every hour after placing the spinner flask into the incubator, the flask was taken out of the incubator and a 0.5 mL sample of the media was removed, and the cells were counted in that sample. The cells present in that sample represent the population of cells that had not been attached yet.

RESULTS AND DISCUSSION

Static Seeding

Effects of Time on Cellular Attachment

Once the cells have properly been seeded onto the scaffold, the amount of cells that actually attach to the scaffold varies significantly over time. There are two distinct regimes that were noticed with respect to the percentage of cells attaching to the scaffold as time elapses. This can be seen in Figure 2. Initially it goes through an exponential phase in which the attachment rate is not proportional to the time that has elapsed from the prior data point. After 3 to 4 hours, it is no longer showing signs of an exponential rate of attachment. It begins to plateau off, which is the second regime that is noticed from Figure 2.

The exponential phase is experienced because initially, there are many easily accessible sites for attachment. Since the only driving forces for the cells to move around on the scaffold are through diffusion or capillary action, the areas of the scaffold that the cells can reach are very limited. This lack of mobility explains why the rate of attachment plateaus off after a certain amount of time. Unattached cells can become clustered around cells that are attached, thus inhibiting the effects of diffusion or capillary action. Although those unattached cells are intercepted by the three-dimensional environment they, will be filtered out and counted as cells that have not attached once the scaffold is washed with PBS.

No matter how much time elapses, the percentage of cells attached will never reach 100% because of two reasons. The first reason is because the cells tend to be clustered together and can be wedged in-between other cells that have attached to the scaffold, thus having no ability to interact with the scaffold. Cells that can not interact

with the scaffold do not have a chance to attach and, as previously stated, are called intercepted cells. Furthermore, immediately after seeding, some cells are lost and do not have the ability to become intercepted or attached cells. These cells are lost due to the scaffold being initially wet in order to assist in a more uniform distribution of cells, by facilitating diffusion of cells throughout the scaffold. Some of the media that the scaffold was initially wetted with was displaced by the solution of cells that were seeded and therefore allowed some cells to be carried out of the scaffold via bulk flow and partially be diffusion into the well below.

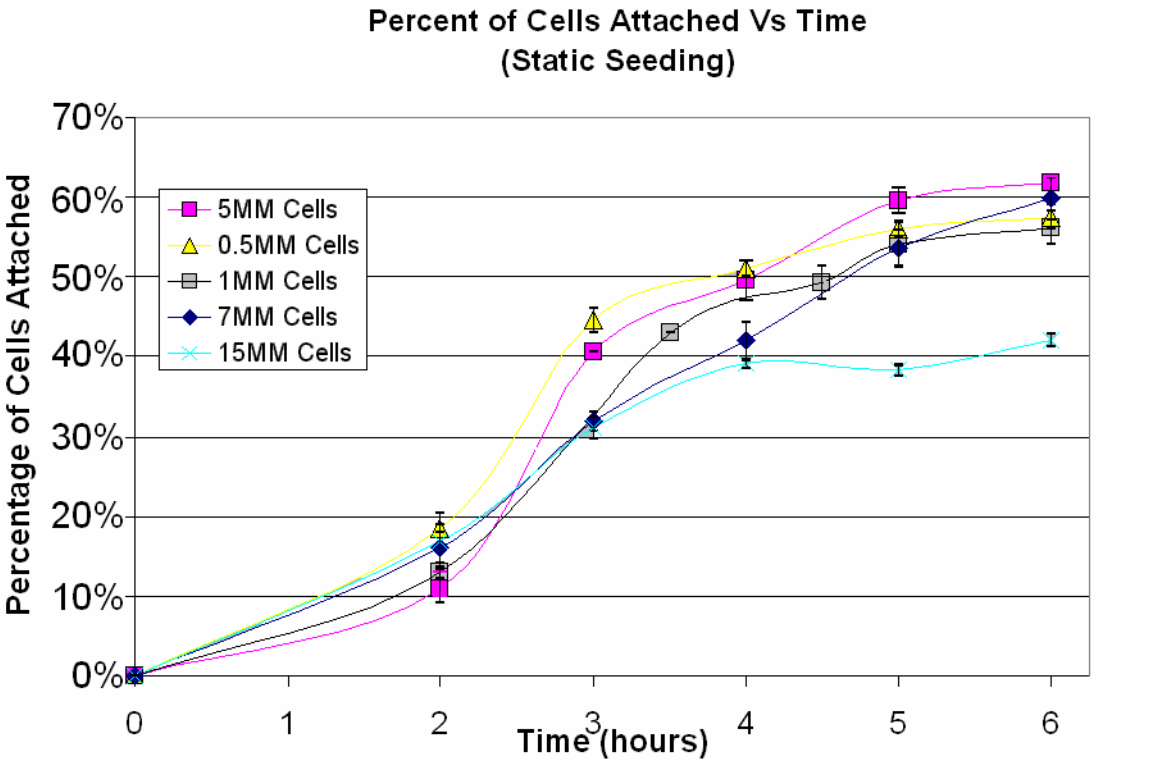

Figure 2: Percent of Cells Attached Vs. Time (Static Seeding)

Although the percentage of attachment were relatively similar within the range of 0.5 and 7 million cells, the number of cells that attached to the scaffold were drastically different. This can be seen in Table 1.

Table 1: Number of Cells Attached per Cubic Inch of Scaffold (Static Seeding)

Cells Seeded (10^6)	Number of Cell/cubic inch of scaffold
0.5	8343490.626
5	90835219.69
1	16251668.7
7	121524696.7
15	182831272.9

The percentage of cells attaching to the scaffold over time can be accurately represented by the first-order kinetics model, **Equation 2**.

$$[C] = [C]_o * e^{-kt} \qquad \textbf{Equation 2}$$

Where t is the time that has elapsed since initially seeding the cells onto the scaffold, [C] is the cell concentration at time t, $[C]_o$ is the initial cell concentration at t = 0, and k is the rate constant of cell attachment

Equation 2 can be made linear by simply making the abscissa $\ln([C]_o/[C])$, while the ordinate can be left as time. Making **Equation 2** linear is advantageous because the slope of the line is the rate constant k. Figure 3 demonstrates that the first order model accurately represents the data from Figure 2.

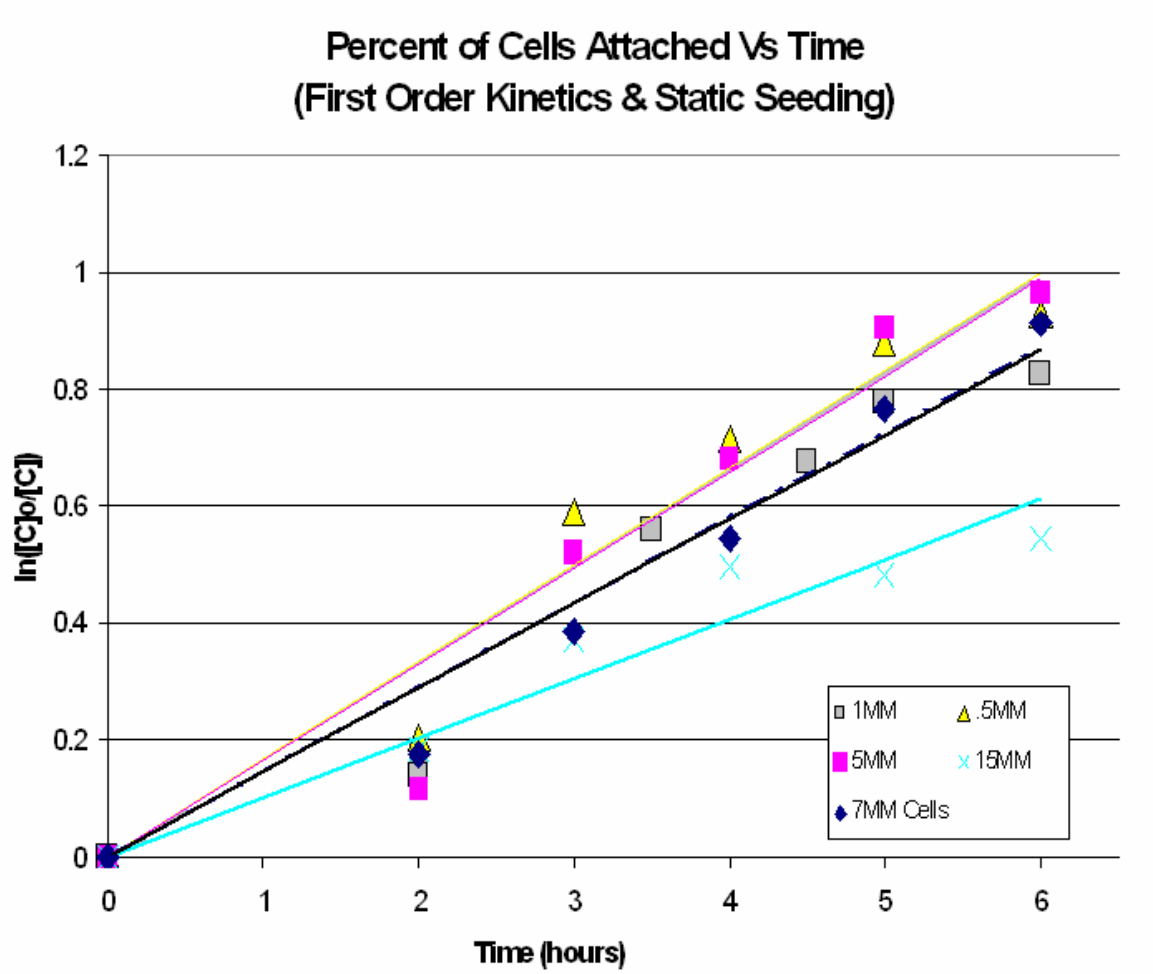

Figure 3: Percent of Cells Attached Vs. Time (First Order Kinetics & Static Seeding)

Effects of Seeding Concentration on Cellular Attachment

Initial seeding concentrations of the cells seeded to the scaffold are significant because it is desired to maximize the total number of cells attached while reducing wasted, unattached cells. The initial seeding concentrations that were tested ranged from 0.5 million to 15 million cells. There are three different outcomes that can occur when the cells are seeded: it can become attached to the scaffold, be intercepted by the scaffold, or be neither attached nor intercepted by the scaffold. Attachment is when the cells adhere to the fibers of the scaffold, and interception is where the cells are simply held in place because of the physical size of the pores on the scaffold. The experiments on initial

seeding concentration led to two conclusions. The first conclusion is that the percentage of cell attachment is not strictly dependent upon the number of cells that were initially seeded but also relied on the time given to attach to the scaffold. Additionally, at highly concentrated seeding concentrations, the seeding efficiency decreases.

In some cases, the time given for the cells to attach to the scaffold has a higher influence than the concentration of cells in the initial seeding. In fact, in the ranges of 0.5 million to 7 million cells, the percentage of cell attachment is independent of the initial seeding concentration. Therefore, the time that the cells are allowed to attach to the scaffold becomes the influential factor for the percentage of attachment in this range. It can be seen in Figure 3, that the trials that had initial seeding concentrations between 0.5 and 7 million cells showed the same pattern. Therefore, the attachment rate for the range of 0.5 to 7 million cells is not dependent on the number of cells introduced to the scaffold, but rather just a function of time.

It was observed that between the ranges of 0.5 million to 7 million cells, the overall attachment rate was around 60%. Utilizing a Tukey-Kramer statistical analysis to compare the means and a significance level of 0.01, it was determined that trials within the range of 0.5 and 7 millions cells are statistically the same. On the other hand, the trials that use 15 million cells as their initial seeding concentration were shown to be statistically less than the other trials. This can be seen in Figure 4, which was generated from JMP. The red circle and numbering only on the 15 million cell trials, in Figure 4, indicates that at a significance level of 0.01, it is not statistically equivalent to the other trials. The trials that used 15 million cells had an attachment percentage of 42%. These percentages can be seen in Figure 2. The statistically lower attachment percentage

signifies that seeding at a concentration of 15 million cells is inefficient and more cells are being wasted than necessary. The 42% attachment of 15 million cells implies that only 6.2 million cells have attached to the scaffold. Those 15 million cells would be better utilized if the cells were split into two equal samples and seeded onto separate scaffolds. Splitting the sample would lead to around a 60% attachment of each of the two scaffolds, thus leading to 9 million cells attaching to a scaffold instead of the 6.2 million cells.

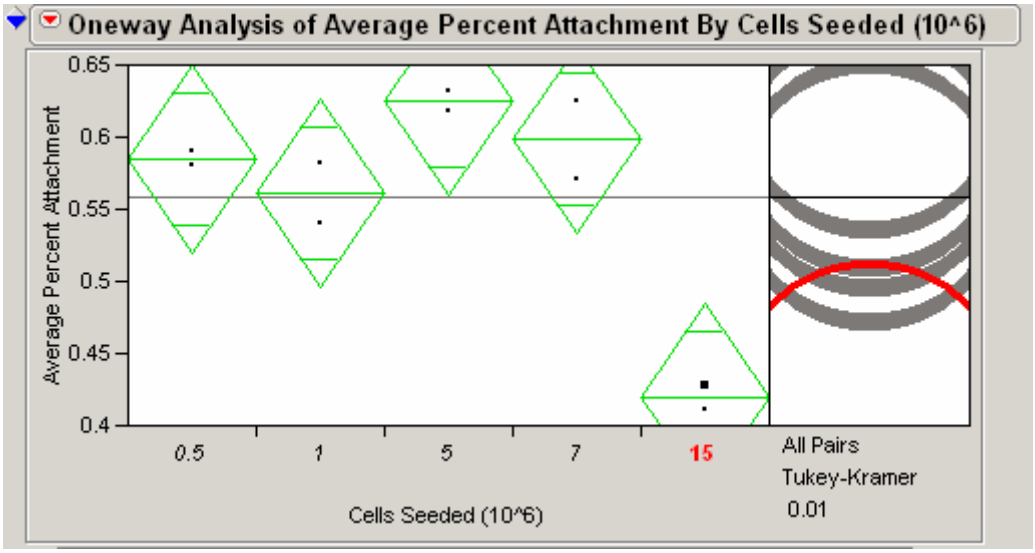

Figure 4: Statistical Analysis of the Significance of Seeding Concentration in Static Seeding

The decrease in the percentage of attachment at 15 million cells is because at higher levels of seeding concentrations, the cells are more likely to clump together and therefore become intercepted by the scaffold rather than attached. Intercepted cells may not become attached due to the lack of interaction with the scaffold itself and therefore will be washed away by the PBS and counted as unattached cells.

The rate of attachment represents how quickly the cells attach over time. The summary of the rates are in Table 2. It can be seen that the trials between 0.5 to 7 million cells have approximately the same attachment rates, but the trials that have 15 million

cells have a significantly reduced rate of attachment. This reduced rate of attachment is due to the increase of intercepted cells at the elevated concentration of 15 million cells.

Table 2: Attachment Rates of Cells at Different Initial Seeding Concentrations

Seeding Concentration	Rate Constant of Attachment (1/hr)
0.5 million cells	0.1667
1 million cells	0.1448
5 million cells	0.1647
7 million cells	0.1443
15 million cells	0.1090

Effects of the Hydrophilic Properties of the Scaffold

Cells have a distinct affinity towards environments that are hydrophilic. This affinity is due to the structure of their cell walls. The cell wall consists of areas that are hydrophilic and areas that are hydrophobic. The part of the cell wall that is hydrophobic is between two hydrophilic layers, thus the area of the cell wall that is exposed to the external environment is hydrophilic and therefore cells should be drawn to more hydrophilic settings. Since it is known that cells desire hydrophilic environments, it is favorable to change the hydrophilic nature of the scaffold[11]. The scaffold can be made more hydrophilic by boiling the scaffold in a 10% NaOH solution for an hour. Boiling the scaffold in the NaOH solution adds functional groups to the fibers of the scaffold, which in turn will increase the scaffold's hydrophilic status. Scaffolds that were treated with the NaOH solution were compared to scaffolds that were not. This comparison will signify the effectiveness of a more hydrophilic environment.

Trials that used the more hydrophilic scaffold showed an average of 56% attachment. Trials with an unaltered scaffold showed an average of 39% attachment. Although the attachment percentages are drastically different from each other, the qualitative structure of the curves is quite similar. These two observations can be seen in

Figure 5. The curve for the treated scaffold seems to just be an upward translation from the non-treated scaffold. Both curves experienced an exponential phase followed by a plateau at relatively the same hours of incubation. The qualitative similarities and quantitative differences lead to the conclusion that a more hydrophilic scaffold creates an environment that the colon cancer cells are more receptive to.

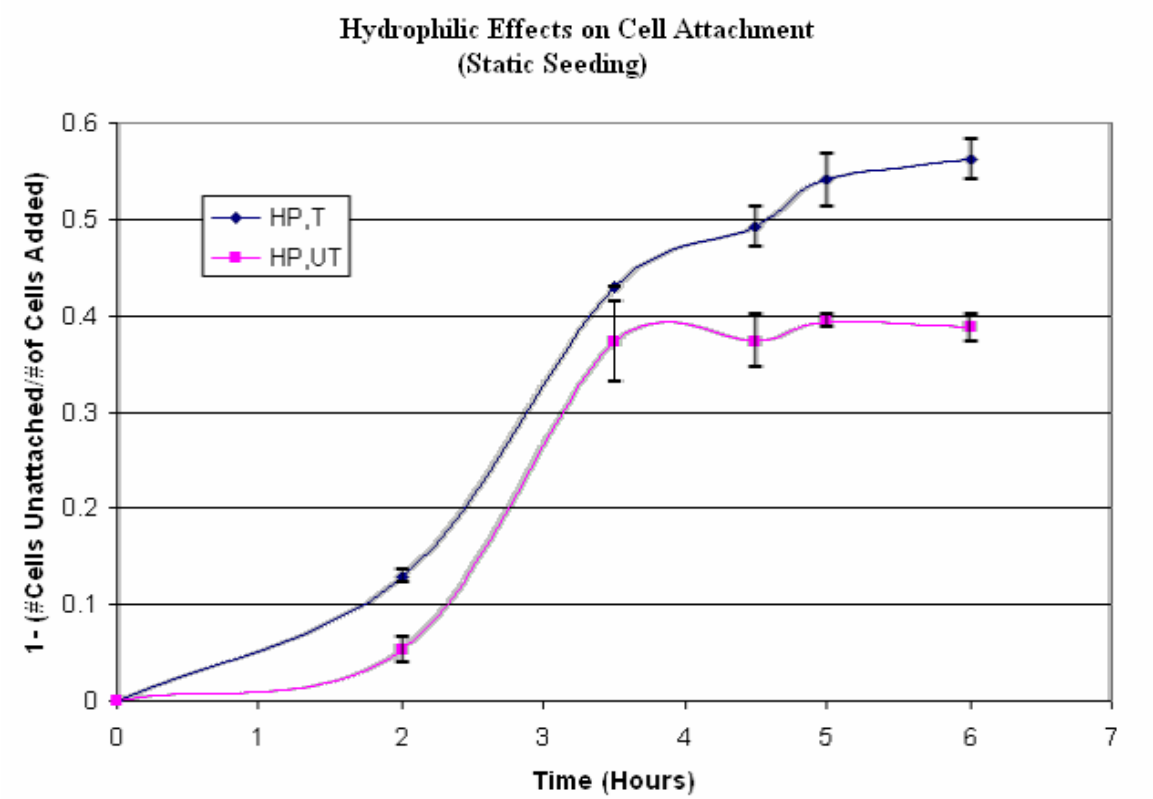

Figure 5: Hydrophilic Effects on Cell Attachment (Static Seeding)

Utilizing statistical analysis tools and the data collected from these trials, a probability of how different the trials between the treated and untreated scaffolds can be calculated. Using a t-statistic and a null hypothesis that the means of the different treatments were statistically equal, it was determined that there is only a 2.02% (p-value equal to 0.0202) chance that it would be a wrong decision to reject the null hypothesis.

The data present in Figure 5 can also be accurately represented by the first-order kinetics model, **Equation 2**. When modeled with **Equation 2,** the data was fit to a line in which the rate of attachment is the slope of that line. The rates of attachment, much like the percentage of cells attached, are significantly higher for the treated scaffold, as seen in Table 3. This increased rate of attachment and increased percentage of attached cells indicate that the treated scaffold allows for more of the colon cancer cells to attach and at a quicker rate.

Table 3: Attachment Rates of Cells an in Hydrophilic Environment

Type Of Scaffold	Rate Constant of Attachment (1/hr)
Treated in NaOH	0.1491
NOT Treated in NaOH	0.1074

Effects of Scaffold Porosity

The porosity of the scaffold is an important parameter to investigate because it directly affects the diffusive properties of the cells through the scaffold and capillary action that occurs in the scaffold. Porosity is defined as the volume of the pores divided by the volume of the material. Since porosity is the ratio of the pores to the material volume, porosity can never be greater than unity. The porosity of the scaffolds was changed by compressing them. The compression process was accomplished by applying force on top of the scaffolds during the autoclave process at 121^{o}C. The width of the scaffold was reduced by about 42%. This is a porosity change from 0.93 to 0.89.

Trials were done with compressed and uncompressed scaffolds to determine the effects of having a smaller pore volume. The trials that used the uncompressed scaffolds, those with larger pore volume, showed an average of 56% attachment. The trials that used the compressed scaffolds showed an average of 42% attachment. The results can be

seen in Figure 6. These trials can also be properly modeled after the first-order kinetics model, **Equation 2**. The rates of attachment for the uncompressed and compressed scaffolds are 0.1448 hr^{-1} and 0.1070 hr^{-1}, respectively. These rates, summarized in Table 4, indicate that the uncompressed scaffold provides an environment that allows for quicker cell attachment. Utilizing a t-statistic, it was determined that if in fact these trials were statistically equivalent, then there would only be a probability of 0.008 of observing this type of dispersion in the data.

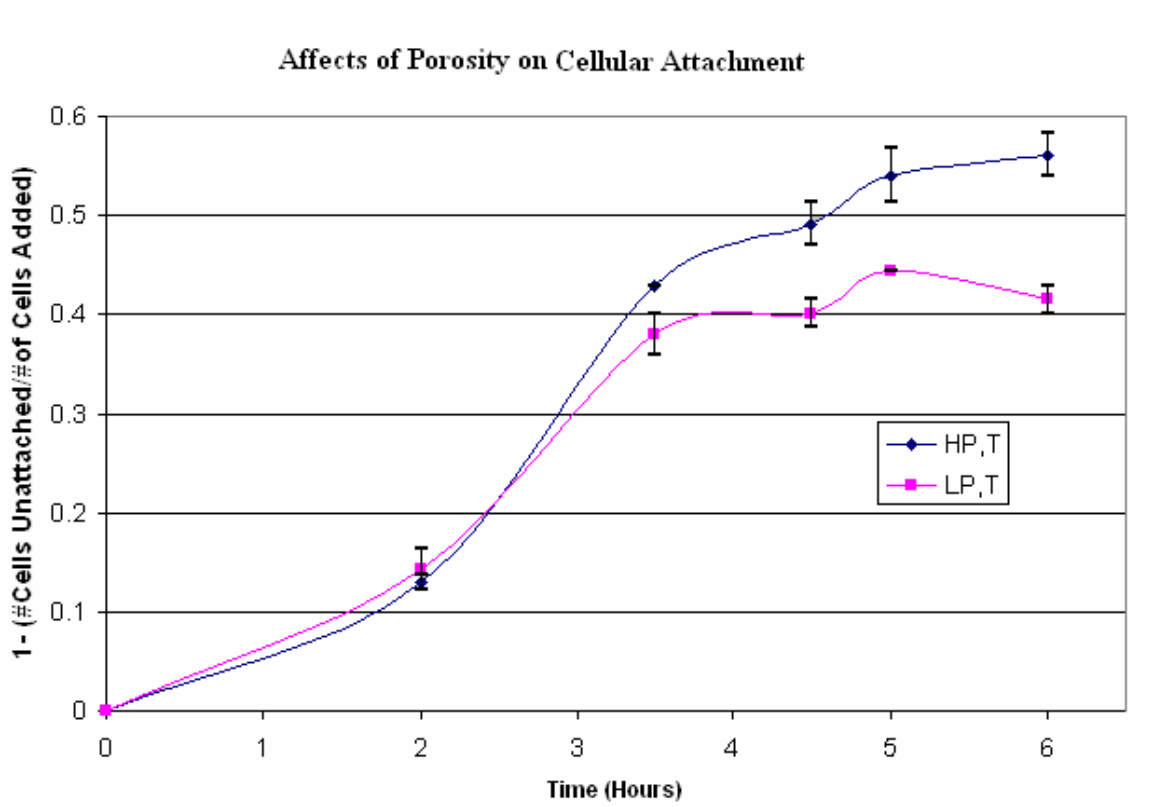

Figure 6: Affects of Porosity on Cellular Attachment

Table 4: Attachment Rates of Cells with Different Porosities of Scaffolds

Scaffold Type	Rate Constant of Attachment (1/hr)
Compressed	0.1448
Uncompressed	0.1070

The results from these trials indicated that an uncompressed scaffold is overall better than an uncompressed scaffold at allowing colon cancer cells to attach at a higher quantity and rate that the cells attach. Attachment percentages are higher in the trials that used an uncompressed scaffold because it reduces the likelihood for cells to be intercepted and it increases diffusion of the cells through the scaffold. Conversely, there is a greater likelihood for cells to be intercepted and remain unattached in the compressed scaffold. This is due to the limited forces of mobility which the cells have in static seeding, the cells can become stuck between the fibers of the scaffold and remain suspended and thus, will not allow for adequate contact area between the cells and the scaffold for proper attachment to occur. This will increase the ratio of intercepted cells to attached cells.

Since the uncompressed scaffolds have larger pathways through which the cells and the solution can pass through, an increase in diffusion can be observed. This will allow cells to explore more of the scaffold and therefore locate ample attachment sites that were previously unavailable to them in a compressed scaffold.

Dynamic Seeding

Along with testing different parameters that pertain to static seeding, dynamic seeding was investigated as well. In dynamic seeding, there was an additional force added to the system to change the hydrodynamics of the media that the cells and scaffold was in, which was not present in static seeding. The additional force was caused by a stir bar that was incorporated into the immediate environment that the scaffold was in. The stir bar provides tangential and radial mixing within the system. Even though tangential and radial mixing are not the ideal flow patterns for the most uniform mixing, it provides ample mixing while maintaining a lower shear stress. Although axial mixing will provide for better re-suspension and mixing of the system, axial mixing is usually accompanied by higher shear stress. A high shear stress is not desired in biological systems because cells can be very delicate and if too much shear stress is applied to the cells, the cells may become damaged or die.

Within the dynamic seeding system, the stir bar causes the suspended unattached cells in the media to be consistently pushed through the scaffold via tangential and radial mixing. This allows the cells to be more mobile and to explore the entire scaffold. Also, this allows the cells to be no longer bounded by diffusion and capillary action, like in static seeding. The higher mobility of the cells allows them to find attachment sites throughout the scaffold that might not have been able to be reached prior to the addition of mixing. Therefore, the attachment rate and percentage should increase from that of static seeding.

Two variables were examined for this seeding technique: the concentrations of colon cancer cells initially seeded and different levels of RPM in the system. Initial seeding concentrations of the cells to the scaffold are important because it is desired to

maximize the total number of cells attached while reducing wasted, unattached cells. Even though the initial seeding concentration is an important parameter to study, the different levels of RPM of the stir bar is equally as important. If the RPM of the stir bar in dynamic seeding is too low, there will be an inadequate amount of circulation so the cells cannot explore the entire matrix. Yet if the RPM is too high, it will not allow enough contact time for the cells to attach to the matrix as it passes through.

Effects of Time and Seeding Concentration on Dynamic Seeding

At the RPM of 80, the range of initial seeding concentrations tested for dynamic seeding was from 18 million to 98 million cells. As time progressed from the initial seeding point, cells continually attached until around 100% attachment was observed. Initially, the cells attached very quickly from hour to hour, but around the 6^{th} hour after seeding, the attachment rate began to slow down and started to plateau off with each successive hour, as shown in Figure 7. The percentage of cells attached increases faster in the beginning because initially, there were many sites for attachment. As cells started to attach to the more favorable areas of the scaffold, the probability of a cell encountering an area of the scaffold that was adequate for attachment drastically decreased. This decrease in the probability of finding an attachment site is indicated by the noticeable asymptotical approach to 100% in Figure 7.

Besides showing the effect that time has on the percentage of cells that attach in a dynamic system, Figure 7 also shows the effect of different seeding concentrations. Even though the range of the tested concentrations was sufficiently wide, all the different concentrations levels showed to have the same trends qualitatively. Although the graphs are qualitatively similar, they are quantitatively different. The quantitative trend that is

observed is that the higher the initial seeding concentration, the higher the percentage of cells attached at any given time. This was observed because as the cell concentrations increase, the probability that a cell will contact and adhere to the fibers are much higher than that of a lower concentration system. For example, suppose that there are 500 sites on a scaffold that have the most ideal settings for cell attachment and proper cellular attachment can occur in less than an hour. If there were only 500 cells introduced to this scaffold, there would only be a one to one ratio of cells to fast attachment sites. As the cells begin to attach, it will become increasingly less probable that a fast acting site will be encountered by a cell and become occupied. Therefore, within the first hour, all the fast acting sites will probably not be occupied. Now suppose instead of introducing 500 cells to this scaffold, 2000 cells were introduced. The ratio of cells to fast acting sites would now be four instead of one. Although the probability that one of the 500 fast acting sites will be encountered by a cell and become occupied will still be decreasing as cells become attached, it will not decease as significantly as in the case where only 500 cells were introduced. In this case, it would be highly probable that the 500 fast acting sites will be occupied by cells after the first hour.

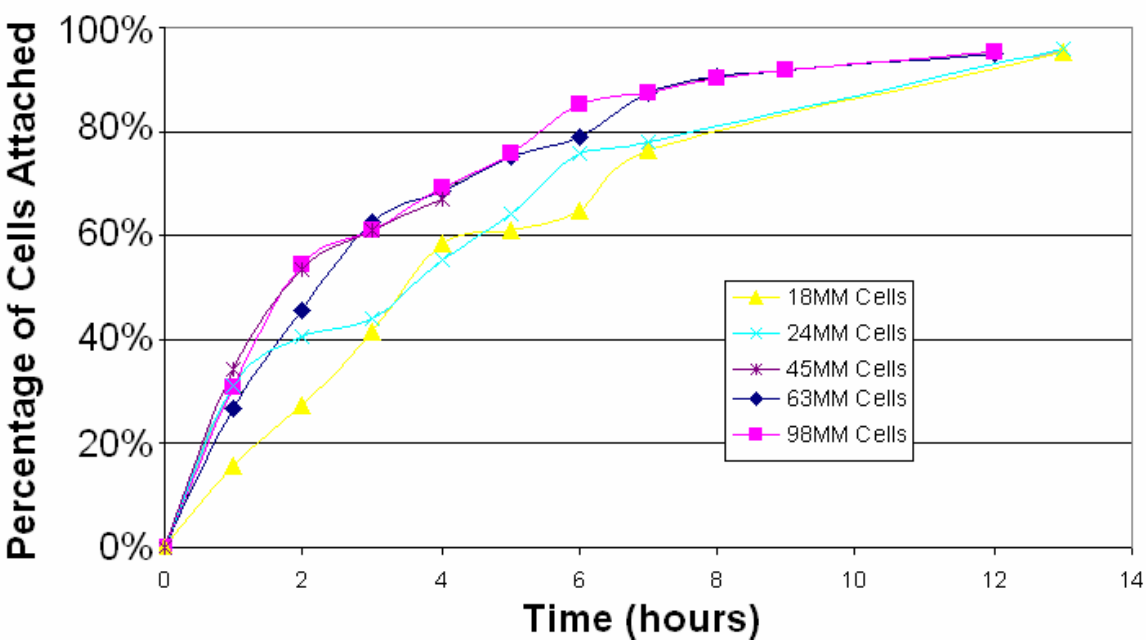

Figure 7: Percent of Cells Attached vs. Time (Dynamic Seeding, RPM = 80)

Although the percentage of attachment all reached a 100%, the number of cells that attached to the scaffold was drastically different. This can be seen in Table 5.

Table 5: Number of Cells Attached per Cubic Inch of Scaffold (Dynamic Seeding)

Cells Seeded (10^6)	Number of Cell/cubic inch of scaffold
18	50281576.83
24	67042102.44
45	125703942.1
63	175985518.9
98	273755251.6

Similar to the static seeding, dynamic seeding at an RPM of 80 also fits the first-order kinetics model, as seen in Figure 8. The rates of attachment, the slopes from the linear regression lines of Figure 8, are summarized in Table 6. It was observed that the higher the initial seeding concentration, the higher the attachment rate becomes.

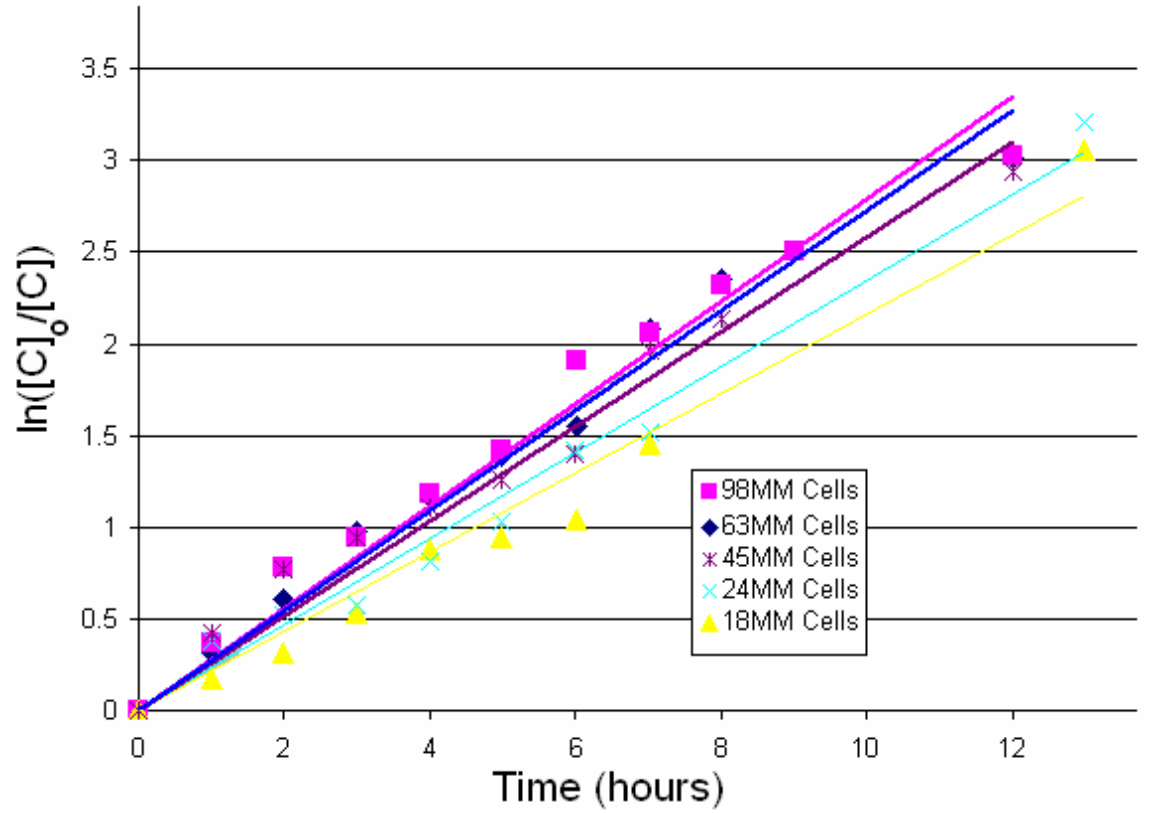

Figure 8: First Order Kinetics Applied to Dynamic Seeding at 80 RPM

Table 6: Rates of Attachment for Dynamic Seeding at RPM = 80

Cells Seeded (10^6)	Attachment Rate Constant (1/hr)
98	0.2794
63	0.2729
54	0.2662
24	0.2340
18	0.2163

Although higher initial seeding concentrations led to higher attachment rates within the range that was tested, extrapolating this data showed that dynamic seeding at a RPM of 80 is asymptotically approaching ~0.285/hr, as seen in Figure 9. This indicates that if further trials were done at higher levels of cells seeded, the highest attachment rate

that would be observed would not exceed ~0.285/hr, but it may lie below this approached rate due to clustering of cells at higher seeding concentrations.

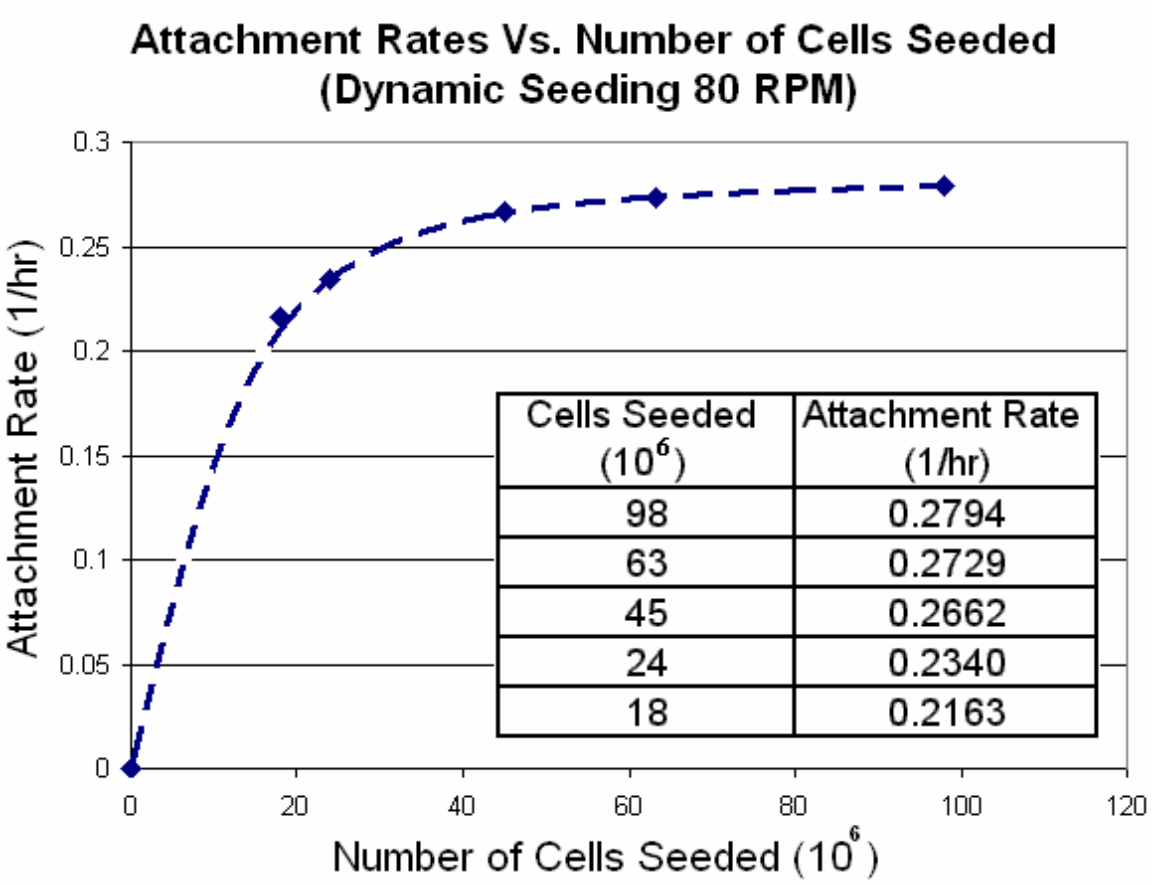

Figure 9: Attachment Rates vs. Number of Cells Seeded (Dynamic Seeding, 80 RPM)

Effects of Changing the Hydrodynamics of the Dynamic System

Besides studying the effects of time and seeding concentrations, the hydrodynamics of the system were also varied to see the effect on cellular attachment. To change the hydrodynamics of the system, the RPM levels of the stir bar were changed. RPM levels that were tested were 80 and 120. The level of RPM is an important parameter to study because it provides the main driving force behind cell mobility in dynamic seeding.

Changing the RPM from 80 to 120 changed many aspects of the system. The major difference that was created by the elevated RPM was that the data no longer could be modeled by first-order kinetics and therefore the rates of attachment of the trials with 120 RPM cannot be compared to those from trials with 80 RPM due to different units for the rate constants. Also, at the elevated RPM of 120, it seems that cellular attachment has become independent of the seeding concentration. This can be seen in Figure 10. Trials that had seeding concentrations that ranged from 22 to 65 million cells at an RPM of 120 are a bit indistinguishable, unlike their counterparts at an RPM of 80.

Another difference that can be noticed in Figure 10 is that the trials with 120 RPM are not monotonically increasing with time, as were the trials with an RPM of 80. This indicates that cells are attaching and then detaching from the scaffold as time progresses. This can be due to the fact that at 120 RPM there is too much shear stress introduced to the system. Although the cells are initially being intercepted or attaching, the elevated shear stress causes some of those cells to become released from the scaffold. In a sense the cells are being pulled off of the scaffold, which is counterproductive to the goal of seeding. Also at higher RPM levels, there is a shorter mean residence time that the cells experience through the scaffold, meaning that the cells spend less time traversing through the scaffold, thus reducing the contact time that the cells have with the scaffold fibers and therefore reducing the likelihood of attachment.

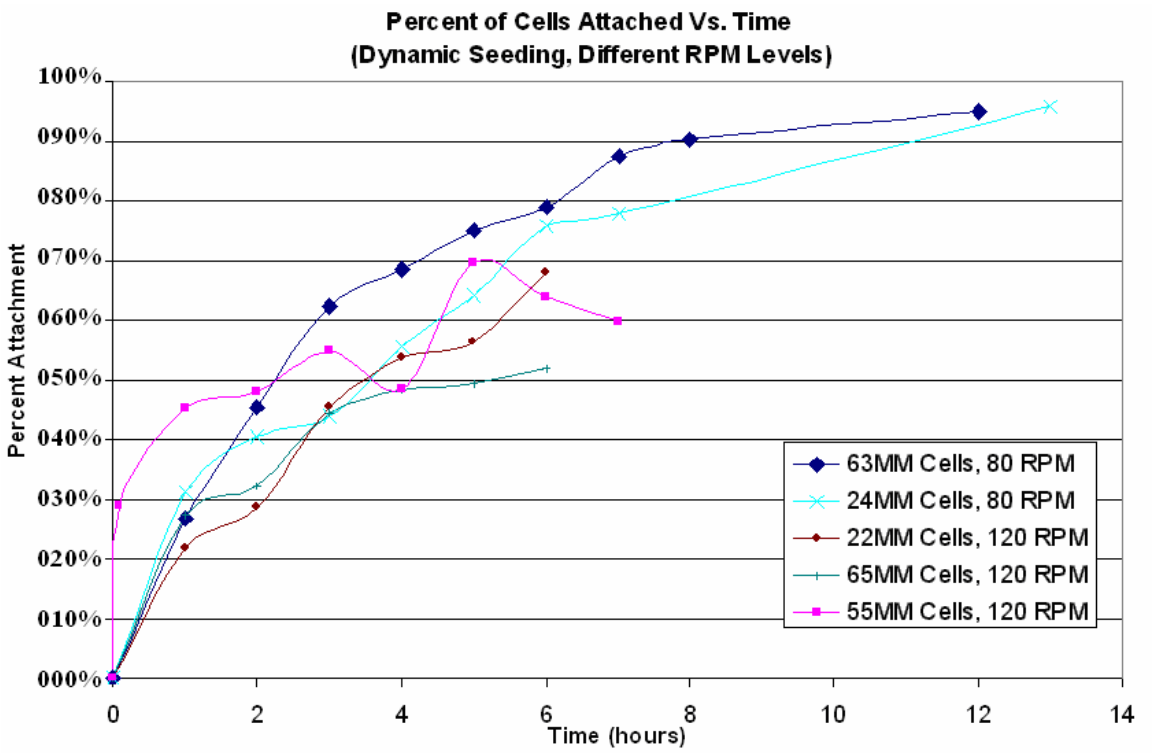

Figure 10: Effects of Different RPM Levels in Dynamic Seeding

CONCLUSIONS

Efficiently seeding colon cancer cells to a PET scaffold to produce an accurate 3D *in vitro* model was the focus of this study. Both static and dynamic seeding methods were investigated. Most the parameters studied for static seeding were properly modeled by first-order kinetics. In static seeding, it was observed that as time elapsed from the point of seeding, the attachment of cells would initially be exponential but then plateau off after six hours. It was determined that the rate of attachment was independent of seeding concentrations within the range of 0.5 to 7 million cells. That rate of attachment that was observed within that range was 0.15 hr^{-1}. Once above this range, the rate of attachment drastically decreased by 33% or more.

In dynamic seeding, it was observed that as time progressed from the introduction of the cells to the system the percentage of cells attached would approach 100%. Initially the percentage of cells attached increased rapidly but after the first 4 hours, the percentage of cells attaching slowed down and approached 100%. Unlike static seeding, dynamic seeding showed an increase in the attachment rate when the initial seeding concentration increased. Although it was observed that there was an increase in the rate of attachment as the initial seeding concentration increased, extrapolating the data indicates that the rate of attachment asymptotically approaches 0.285 hr^{-1}. Therefore, the rate of attachment becomes independent of seeding concentrations at highly concentrated seeding. It was also observed that when the RPM of the system was increased, cellular attachment was reduced due to the increased shear stress supplied by the increase in the RPM.

Although the main focus was on the different seeding methods, scaffold properties were also investigated. It was determined that if the scaffold was not compressed and was treated with a 1% NaOH solution, then the scaffold showed a 140% increase in both the quantity of cells that attached and in the rate of attachment of cells. This increase was due to the larger pores in the scaffold which facilitated diffusion and the addition of hydrophilic functional groups onto the scaffold.

NOTATIONS

[C] = cell concentration at time t
$[C]_o$ = initial cell concentration, t = 0
c = sum of cells counted in the corner cells of the hemocytometer
D = dilution rate
HP = High porosity scaffold
LP = Low porosity scaffold
k = rate of cell attachment
MM = million
N = total number of cells in sample, determined by counting with a hemocytometer
t = time
T = a scaffold treated in NaOH
UT = a scaffold that was not treated with NaOH
V_s = Volume of sample that being counted for the number of cells

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